Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Testing Enterotoxigenic (987P Pilus) *Escherichia coli* Bacterins

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1. Introduction

This Supplemental Assay Method (SAM) for potency testing inactivated *Escherichia coli* bacterins employs a capture enzyme-linked immunosorbent assay (ELISA) for 987P pilus antigen. Relative potency is determined by comparing the 987P antigen content of the test bacterin to the 987P antigen content present in a nonexpired, suitably qualified reference bacterin.

2. Materials

2.1 Equipment/instrumentation

(Unless specified, equipment vendor is optional.)

2.1.1 Microplate reader with dual wavelengths (490 nm and 650 nm)

2.1.2 Automatic microplate washer (optional)

2.1.3 Micropipettors, to cover the range of 5.0 µl to 1000 µl

2.1.4 8- or 12-channel micropipettor, to cover the range of 50 µl to 200 µl

2.1.5 Orbital shaker

2.1.6 Balance, validated from 150 mg to 15 g

2.1.7 Relative Potency Calculation Software (United States Department of Agriculture, Veterinary Services, Center for Veterinary Biologics-Laboratory [CVB-L]), current version

2.2 Reagents/supplies

2.2.1 96-well flat-bottom microtitration plates (Immulon 2; Dynatech Laboratories, Inc. or equivalent)

2.2.2 96-well microtitration plates suitable for making serial dilutions (transfer plates)

2.2.3 Plate sealers

2.2.4 Carbonate coating buffer

2.2.5 Phosphate-buffered saline (PBS), pH 7.2

2.2.6 Phosphate-buffered saline with 0.05% Tween 20 (PBS-Tween 20)

2.2.7 PBS-Tween 20 with 2.0% bovine albumin fraction V (monoclonal antibody [MAb] diluent)

2.2.8 PBS-Tween 20 with 1.0% normal rabbit serum (conjugate diluent)

2.2.9 Phosphate elution buffer (optional)

2.2.10 Sodium citrate for antigen elution (optional)

2.2.11 Sodium desoxycholate elution buffer (optional)

2.2.12 Citrate buffer

2.2.13 o-phenylenediamine

2.2.14 Hydrogen peroxide, 30%, stabilized

2.2.15 Stop solution

2.2.16 987P-specific antigen-capture polyclonal antibody (987P PAb), rabbit origin. Obtain the 987P PAb from the CVB-L.

2.2.17 987P-specific antigen-detection monoclonal antibody (987P MAb), mouse origin. Obtain the 987P MAb from the CVB-L.

2.2.18 Horseradish peroxidase-conjugated goat anti-mouse IgG (H+L). Obtain this reagent from a commercial source (Jackson ImmunoResearch Laboratories, Inc., 115-035-062 or equivalent).

2.2.19 Test bacterin(s) containing 987P antigen

2.2.20 Reference bacterin containing 987P antigen (must be approved by the Animal and Plant Health Inspection Service)

2.2.21 Water, deionized or distilled, or water of equivalent purity

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware; automated microplate washer and reader; and data recording and evaluation software. They need specific training in the performance of this assay.

3.2 Preparation of equipment/instrumentation

3.2.1 Operate and maintain all equipment according to manufacturers' recommendations.

3.2.2 Validate the ELISA microplate reader according to the current version of GDOCSOP0005.

3.3 Preparation of reagents/control procedures

Caution: Concentrated solutions of acids and bases are used to prepare some of the following reagents. Both are hazardous and must be handled properly. Consult Material Safety Data Sheets (MSDS) (current version) for proper safety procedures.

3.3.1 Carbonate coating buffer--NVSL media 20034

Na_2HCO_3	0.159 g
$NaHCO_3$	0.293 g
Water	q.s. to 100 ml

Adjust pH to 9.6 ± 0.1. Store at 2°-7°C up to 1 wk.

3.3.2 Phosphate buffered saline--NVSL media 10559

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Water	q.s. to 1 L

Adjust pH to 7.2 ± 0.1. Store at room temperature (20°-25°C). If long-term storage (up to 6 mo) is desired, autoclave to sterilize.

3.3.3 Phosphate Buffered Saline with 0.05% Tween 20--NVSL media 30179

PBS (See **3.3.2**)	1 L
Tween 20	0.5 ml

Store at 20°-25°C no longer than 6 mo.

3.3.4 PBS-Tween 20 with 2.0% bovine albumin fraction V (MAb diluent)

PBS-Tween 20 (see **Section 3.3.3**)	25 ml
Bovine albumin fraction V (Scientific Protein Laboratories, Viobin Corp., Waunakee, WI, 40-6197-2-1160 or equivalent)	0.5 g

Prepare immediately prior to use. Swirl gently to dissolve the powder.

3.3.5 PBS-Tween 20 with 1.0% normal rabbit serum (conjugate diluent)

PBS-Tween 20 (see **Section 3.3.3)**	25 ml
Normal rabbit serum	0.25 ml

Prepare immediately prior to use. Swirl gently to mix.

3.3.6 Phosphate elution buffer

KH_2PO_4 (reagent grade)	8.2 g
Water	94 ml

Adjust pH to 9.3 ± 0.1, or other appropriate pH as optimized for use with a specific bacterin. Store at 20°-25°C no longer than 1 mo.

3.3.7 Sodium desoxycholate elution buffer

Na desoxycholate (reagent grade)	0.50 g
PBS (see **Section 3.3.2**)	100 ml

Store at 2°-7°C for up to 30 days. Warm to 20°-25°C prior to use. (The buffer gels at 2°-7°C.)

3.3.8 Citrate buffer (pH 5.0)--NVSL media 20033

Citric acid (reagent grade)	5.26 g
Na_2HPO_4-$7H_2O$	6.739 g
Water	q.s. to 1 L

Adjust pH to 5.0 ± 0.1. Store at 2°-7°C up to 60 days. Use to prepare substrate solution (**3.3.9**)

3.3.9 Substrate solution (quantities for 1 plate)

Citrate buffer	12 ml
o-phenylenediamine (OPD) (Sigma P6787 or equivalent)	4 mg
30% H_2O_2 (stabilized)	5 µl

Prepare within 15 min of use.

Caution: o-phenylenediamine is a carcinogen. See appropriate MSDS for precautions when handling this product.

3.3.10 Stop solution (2.5 M H_2SO_4)--NVSL Media 30171

Concentrated (98%) H_2SO_4	10 ml
Water	90 ml

Add acid to water. Store at 20°-25°C up to 5 yr.

3.3.11 Bacterins containing 987P antigen

3.3.11.1 Reference bacterin

3.3.11.2 Test bacterin

CRITICAL CONTROL POINT: Ideally, the reference and test bacterins should be produced by the same Outline of Production. If reference formulation differs from that of the test bacterin, the assay must be validated to show that this does not adversely affect assay performance or accuracy of results.

3.4 Preparation of the sample

3.4.1 Antigen-elution treatments

Many bacterins do not require antigen-elution treatment prior to being serially diluted in twofold increments with PBS-Tween 20. Test representative batches of each adjuvanted product with and without each antigen-elution treatment to determine if the treatment specifically enhances the 987P antigen capture. If no enhancement of the 987P antigen capture can be demonstrated, test the bacterins without antigen-elution treatment. Treat the reference bacterin and the test bacterins by the same elution procedure. Alternative elution procedures, other than those described here, may be more appropriate for some bacterins.

3.4.1.1 Aluminum-adjuvanted bacterins

Bacterins adjuvanted with aluminum hydroxide may be treated with either sodium citrate or phosphate elution buffer prior to making serial twofold dilutions in PBS-Tween 20.

1. Sodium citrate elution

Add 1.00 g sodium citrate to 10 ml of aluminum hydroxide-adjuvanted bacterin (10% w/v). Place on an orbital shaker (100-120 rpm) overnight at 36°-38°C. Consider treated bacterin to be undiluted.

2. Phosphate buffer elution

Add 1.0 ml of phosphate elution buffer to 1.0 ml of aluminum hydroxide-adjuvanted bacterin. Place on an orbital shaker (100-120 rpm) overnight at 36°-38°C. Consider treated bacterin to be diluted 1:2.

3.4.1.2 Oil-adjuvanted bacterins

Mix 1.0 ml of the sodium desoxycholate elution buffer with 1.0 ml of bacterin. Place on an orbital shaker (100-120 rpm) overnight at 36°-38°C. Consider treated bacterin to be diluted 1:2.

4. Performance of the test

4.1 Dilute the 987P PAb to the current use dilution in cold carbonate coating buffer, and place 100 µl in each well of 96-well flat-bottom microtitration plates. Seal coated plates with plate sealers. Incubate coated plates overnight at 2°-7°C. Store sealed plates at 2°-7°C no longer than 5 days.

4.2 Make twofold dilutions of reference and test bacterins, using PBS-Tween 20 as a diluent. Add 125 µl PBS-Tween 20 to each well of a clean microtitration plate (transfer plate). Place 125 µl of bacterin in the first well of each row. Test each bacterin in at least 2 replicate rows. Test the reference bacterin and the test bacterin on the same plate.

Use a multichannel pipetting device to make serial twofold dilutions of each bacterin across the plate (125 µl transfer volume). Reserve at least 2 unused wells on each plate to serve as blanks.

The use of at least 7 serial twofold dilutions per bacterin is recommended. Ideally, the selected bacterin dilutions should delineate the sigmoid curve from antigen saturation

to antigen extinction for each bacterin. The dilutions used for the reference and the test bacterin may differ.

4.3 Wash the coated ELISA plates 3 times with PBS-Tween 20. An automatic plate washer (250 µl/well, 40-second soak cycle) may be used. Alternatively, plates may be washed by hand. Tap the plates upside down on absorbent material to remove residual fluid.

4.4 Use a multichannel pipetting device to transfer the bacterin dilutions from the transfer plates to the coated ELISA plates (100 µl/well). Seal the ELISA plates and incubate them on an orbital shaker (100-120 rpm) for 30 min (± 5 min) at 20°-25°C.

4.5 Wash the ELISA plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.6 Dilute the 987P MAb in MAb diluent to the appropriate use dilution and add to each well (100 µl/well). Seal the ELISA plates and incubate on an orbital shaker (100-120 rpm) for 30 min (± 5 min) at 20°-25°C.

4.7 Wash the ELISA plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.8 Dilute the peroxidase-conjugated goat anti-mouse IgG in conjugate diluent to the appropriate use dilution and add to each well (100 µl/well). Seal the ELISA plates and incubate on an orbital shaker (100-120 rpm) for 30 min (± 5 min) at 20°-25°C.

4.9 Wash the ELISA plates 3 times with PBS-Tween 20 as in **Section 4.3**.

4.10 Add substrate solution to each well (100 µl/well). Incubate the ELISA plates on an orbital shaker (100-120 rpm) for 10 min (± 5 min) at 20°-25°C.

4.11 Stop the substrate color development by adding 100 µl stop solution to each well.

NOTE: The OPD substrate buffer undergoes a color shift from yellow to orange when stop solution is added.

4.12 Read the ELISA plates using an ELISA reader with dual wavelengths (490 nm test, 650 nm reference). Calculate the mean absorbance for the blank wells. Subtract the mean absorbance of the blank wells from each bacterin test well absorbance value prior to data analysis.

5. Interpretation of the test results

5.1 Relative potency calculation method

5.1.1 Use the current version of the *Relative Potency Calculation Software* (RelPot) to calculate the relative potency of the test bacterin as compared to that of the reference bacterin.

5.1.2 Do not use bacterin dilutions with mean absorbance values <0.05 (after subtraction of the mean absorbance of the blank) in the relative potency calculations.

5.1.3 Do not use regression lines with slopes > -0.150 in the relative potency calculations. Enter a minimum slope assay parameter of 0.150 in the RelPot spreadsheet in place of the 0.000 default.

5.1.4 Enter the reference and test bacterin data, and execute the RelPot program as outlined in MVSAM0318 (current version).

5.1.5 Report the highest relative potency value (RP value) included in the top scores from each test as the RP for the test bacterin.

5.2 Requirements for a valid assay

5.2.1 An assay must meet the validity requirements of MVSAM0318 (current version) to be considered valid.

5.2.2 Lines determined by first-order linear regression must have a correlation coefficient (r) of ≥0.95.

5.2.3 The reference line and the test bacterin line must show parallelism (slope ratio ≥0.80).

5.2.4 Assays that are not valid may be repeated up to a maximum of 3 times. If a valid assay cannot be achieved with 3 independent assays, the test bacterin is unsatisfactory.

5.3 Requirements for a satisfactory test bacterin

5.3.1 To be considered satisfactory, a test bacterin must have an RP value of ≥1.0. Test bacterins with RP values <1.0 on a valid assay may be retested by conducting two independent replicate tests in a manner identical to the initial test. If both retests are valid and the reported RP values of both of the retests are ≥1.0, the test bacterin is satisfactory.

6. Reporting of test results

Report the results of the test(s) as described in the current version of BBSAM0020.

7. Summary of revisions

This is a new SAM. The information contained in this document was previously available as a DRAFT SAM dated June 22, 1995. This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.